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16. ABSTRACT

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Quantitative ^{15}N NMR Spectroscopy

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Line intensities in ^{15}N NMR spectra are strongly influenced by spin-lattice and spin–spin relaxation times, relaxation mechanisms and experimental conditions. Special care has to be taken in using ^{15}N spectra for quantitative purposes. Quantitative aspects are discussed for the ^{15}N NMR of molecules with different nitrogen functional groups and also mixtures of nitrogen-containing compounds. It is shown that, in general, quantitative data are obtainable from integration of ^{15}N lines in proton decoupled ^{15}N NMR spectra using NOE suppression. Addition of paramagnetic relaxation reagents (PARR) *under controlled conditions* is frequently needed to accomplish the experiment within reasonable time limits.

INTRODUCTION

In spite of its low natural abundance (0.36% vs 1.1% for ^{13}C) and much lower sensitivity (about 2% that of ^{13}C at constant field and at natural abundance) ^{15}N NMR spectroscopy is fast developing as a powerful spectroscopic method.[1] A negative magnetic moment and consequent negative nuclear Overhauser effects (NOEs), coupled with competition among several relaxation mechanisms, result in difficult proton decoupled ^{15}N experiments, with results far more variable than in ^{13}C NMR.[2] The maximum NOEF obtainable with proton noise-decoupling has the value of −4.93, leading to enhanced inverted signals, but when the NOE enhancement factor has a value of 0 to −2, partial to complete loss of signal intensity results.[1,3] Finally, the variability of ^{15}N T_1 values, which can extend to over 100 s, poses great difficulty to the experimentalist in choosing the optimum conditions for any experiment.[4] These difficulties place restrictions on the use of techniques which have been successfully employed with other less abundant nuclei, such as ^{13}C.

In quantitative ^{15}N NMR two cases are considered: first, that of determination of the correct number of various types of N atoms within the molecule. Using modern instrumentation ^{15}N spectra can be obtained from biomolecules with a large number of different N atoms. The intensities of individual lines can, however, vary quite strongly.[5] The second is that of analysis of mixtures of nitrogen-containing compounds. Although better signal-to-noise ratios can generally be obtained from ^{1}H or ^{13}C spectra, these spectra are frequently too complicated for unambiguous separation of lines of individual compounds. In these cases the simplicity of ^{15}N NMR spectra outweighs the signal-to-noise considerations inherent for ^{15}N nuclei.

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The present investigation on individual compounds and mixtures with representative types of different ^{15}N nuclei is aimed towards evaluating methods for producing quantitative natural abundance ^{15}N NMR spectra. Paramagnetic relaxation reagents (PARRs)[6] were used in order to lower the time required to obtain spectra with adequate signal-to-noise ratios. The use of PARRs in ^{13}C and ^{15}N spectroscopy, however, is not without difficulties. Attempts to produce quantitative ^{13}C spectra with chromium PARRs may fail due to residual ^{13}C—^{1}H dipolar relaxation which is not replaced by electron–nuclear dipolar interactions.[7] In ^{15}N NMR spectroscopy, careless use of PARRs may be particularly damaging to experiments, as a result of the ^{15}N spin relaxation characteristics and the site basicity of many nitrogen-containing functional groups.

EXPERIMENTAL

Natural-abundance FT ^{15}N NMR spectra were measured at 27.36 MHz in 15 mm diameter tubes on a quadrature detection Bruker HX-270 superconducting NMR spectrometer interfaced to a Nicolet 1089 computer, or at 15.2 MHz in 25 mm diameter tubes on the Seminole superconducting spectrometer interfaced to a Nicolet 1085 computer. Both computers were modified for termination of data acquisition at points predetermined by channel selection. This was essential for avoiding NOE build-up during gated decoupling experiments, with comparatively narrow spectral windows. (Slow data rates give long data acquisition times with typical 4–8 K data points). T_1 measurements were performed by the fast inversion-recovery method,[8] using an on-line three-parameter exponential fitting program.[9] Some of the T_1 data were processed using a faster version of the exponential fit, implemented on a Data General Eclipse S/130 computer.

Data on the composition of measured samples, experimental conditions, relaxation times, peak heights and integral intensities are given in Table 1.

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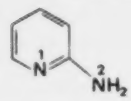
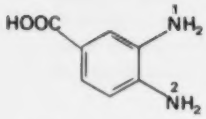
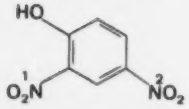
Table 1. Experimental conditions, ^{15}N spin lattice relaxation times and line intensities on measured compounds and mixtures

Sample	Characteristics	Experimental conditions[a] Repetition rate (s)	Total time (h)	Decoupling mode[b]	Relaxation parameters T_1(s) 1	2	3	−NOEF 1	2	3	Experimental ratios ($^1N:^2N$, $^1N:N^3$) Peak heights	Integrals
1a	 5M in acetone	5	0.6	D	33	13		1.2	5.0		<0.05	<0.05
		10	1.4	D							0.06	
		30	6.7	G							1.1	1.0
1b	$+6.10^{-2}$ M	5	0.7	D	4.8	3.3		0.2	1.0		>10	>10
	$Cr(acac)_3$	5	0.7	G							0.9	1.1
1c	same + 1.10^{-3} M	5	0.7	D	1.5	4		0	1.5		−1.0	2.0
	$Gd(dpm)_3$	5	0.7	G							0.5	1.0
2	 1M in DMSO	5	0.5	D	0.9	0.9		5	5		1.4	0.95
3	 1.4 M in DMSO	30	6	G		>100		~0	~0		~1	~1
4	CH_3C^1N, $HCO^2N(CH_3)_2$, n-Bu^3NH_2, equimolar mixture + 5.10^{-2} M $Cr(acac)_3$	5	1	G	7.2	5.0	4.0				0.97 1.03	1.0 0.9
5	$Et_3{}^1N$, $(CH_2)_4{}^2NH_2HO(CH_2)_2{}^3NH_2$, equimolar in C_6D_6 + 6.10^{-2} M $Cr(acac)_3$	5	1.1	G	4.5	1.6	0.6				1.05 1.10	0.95 1.0
6a	$HO(CH_2)_2{}^1NH_2$, $HO(CH_2)_3{}^2NH_2$, $HO(CH_2)_4{}^3NH_2$ equimolar mixture,	20	2	D	1.4	1.9	2.1	1.9	2.7	3.8	0.20 0.15	0.40 0.25
	bulk grade	20	2	G							0.65 0.70	0.95 0.95
6b	$HO(CH_2)_2{}^1NH_2$, $HO(CH_2)_3{}^2NH_2$, $HO(CH_2)_4{}^3NH_2$ equimolar mixture,	20	2	D				2.3	4.3	5.0	0.35 0.25	0.55 0.40
	'Chelex' treated	20	2	G							0.95 0.95	1.05 1.0
7	$OH(CH_2)_2{}^1NH_2$, $HO(CH_2)_3{}^2NH_2$, equimolar mixture, freshly	30	0.3	D	3.6	3.1		4.9	5.0		1.0	1.0
	distilled	30	0.3	G								
8	$CH_3CHNH_2CHNH_2CH_3$	50	0.5	D	5.6	4.6		4.3	2.8		1.7	1.5
	meso : *rac* = 1 : 1	50	0.5	G							1.0	1.20
9	$(CH_3CHNH_2CHNH_2CH_3).2HCl$ *meso* : *rac* = 1 : 1 both 1.3 M in H_2O	50	0.5	D				5.0	5.0		1.0	1.0

[a] Samples 1,5,8 and 9 were measured at 27.4 MHz, samples 2, 3, 4, 6 and 7 at 15.2 MHz. 90° Pulse angles were used except for samples 1a, 3 and 4(30°) and sample 5(45°).
[b] D—{^{1}H} ^{15}N Double resonance with NOE, G—{^{1}H} ^{15}N double resonance without NOE (gated).

RESULTS AND DISCUSSION

Three compounds with nonequivalent nitrogen atoms were studied: 2-aminopyridine, 3,4-diaminobenzoic acid, and 2,4-dinitrophenol. 2-Aminopyridine was chosen as a test compound because of the presence of two different nitrogen atoms and due to its high solubility in acetone. Results of ^{15}N FT NMR experiments on this molecule are depicted in Figs 1 and 2.

Figure 1(a) shows the ^{15}N spectrum of 2-aminopyridine obtained in 0.6 h with continuous wide-band proton decoupling and employing 30° pulses at a 5 s repetition rate. In this case, the NH_2 resonance, having a shorter T_1(13 s) but, more importantly, a large negative NOE appears with a high signal-to-noise ratio. However, the pyridine nitrogen (T_1 = 33 s) is not evidenced. When the pulse repetition time is increased to 10 s, the latter resonance just appears [Fig. 1(b)]. These results are not atypical for ^{15}N spectroscopy. In order to obtain more nearly comparable responses it is necessary to use gated decoupling (to suppress the *small* negative NOEF for the pyridine nucleus) *and* longer pulse intervals. With a pulse interval of 30 s and gated decoupling, the spectrum in Fig. 1(c) results. This required a total of 6.7 h. Competition from nondipolar relaxation (presumably chemical shift anisotropy[10]) in the case of the pyridine nitrogen results in the nearly nulled ^{15}N{^{1}H} signal response even with relatively long (10 s) pulse intervals (the pyridine nitrogen NOEF was measured to be c. −1.2).

Addition of 6×10^{-2} M $Cr(acac)_3$ to 2-aminopyridine lowers the pyridine and NH_2 nitrogen T_1

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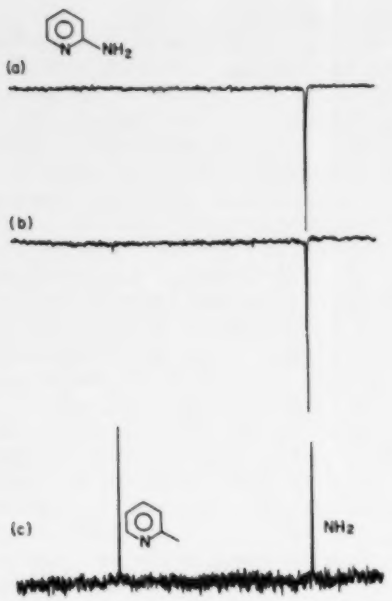

Figure 1. Natural abundance ^{15}N NMR spectrum of 5 molar 2-aminopyridine, conditions as shown. In each case 30° pulse flip angles were used: (a) 0.6 h, 500 scans, 5 s pulse interval; (b) 1.4 h, 500 scans, 10 s pulse interval; (c) 6.7 h, 800 scans, 30 s pulse interval with NOE suppression (gated decoupling).

values to 4.8 and 3.3 s., respectively. Under conditions of continuous wideband proton decoupling the sensitivity of the ^{15}N NMR experiment can be adversely affected with PARR addition. For example, in this case the measured T_1 values show 70–80% replacement of ^{15}N—^{1}H dipolar relaxation. The pyridine resonance should show a residual NOEF near −0.2. However, the NH_2 NOEF will be close to −1.0. Figure 2(a) shows that in this experiment the NH_2 resonance is effectively nulled.

Clearly, addition of $Cr(acac)_3$, even at high concentrations (solubility limits range from 1×10^{-2}–0.1 M), does not ensure effective quenching of ^{15}N{^{1}H} NOEs. This was pointed out previously in ^{13}C NMR.[7] Both $Cr(acac)_3$ *and* gated decoupling should be used to effectively suppress NOEs in accurate quantitative ^{13}C experiments. The critical importance of using both gated decoupling and effective paramagnetic agents in ^{15}N NMR is clearly demonstrated in Fig. 2(a).

The same PARR-2-aminopyridine sample was used to obtain the spectrum shown in Fig 2(b). Note that the total time required to obtain the spectrum in Fig. 2(b) was 0.7 h, far less than for the diamagnetic sample [Fig. 1(c)].

Chromium(III) reagents are outer sphere PARRs, affecting basic and nonbasic nitrogen sites similarly. By contrast, lanthanide PARRs such as $Gd(dpm)_3$ may co-ordinate directly to basic nitrogen sites giving rise to NMR spin-labeling effects.[3] The resulting variations in T_1^e terms may adversely affect attempts to obtain quantitative or even meaningful ^{15}N spectral data in the presence of continuous proton decoupling. Figure 2(c) shows the ^{15}N spectrum obtained with continuous proton decoupling from 5M 2-aminopyridine with 1×10^{-3} $Gd(dpm)_3$. In this case, the pyridine signal NOEF is essentially quenched while the NH_2 resonance NOEF is approximately −1.5. The T_1 values measured for this sample were 1.5 s for the pyridine nitrogen and 4 s for the amine nitrogen. (The more basic pyridine site is indicated by the differential in the T_1 values). This corresponds to 95% replacement of diamagnetic relaxation for the pyridine nitrogen but only ~70% replacement for the NH_2 nitrogen.

Gated decoupling again provides a satisfactory result, used this time in conjunction with $Gd(dpm)_3$; Fig. 2(d) shows this spectrum. It should be noted that the basic-site pyridine resonance is substantially broadened by the PARR.

One further caution is in order. Even 'inert' chromium outer-sphere relaxation reagents can show site or molecular selectivities resulting from ligand H-bonding, steric, or even translational diffusion effects.[11] For accurate quantitative NMR studies using these relaxation reagents, it is sometimes necessary to use quite conservative pulse conditions (relatively long pulse intervals) to allow for moderate variation in T_1 values within the sample.

In 3,4-diaminobenzoic acid (2) similar relaxation characteristics are anticipated for both amino groups. Preferred anisotropic motion about the axis connecting the carboxyl and *p*-amino groups has no observable effect on *para*-NH_2 relaxation because the N—H bond vectors are not aligned on this preferred axis.

Indeed, both amino groups have full NOEs and identical T_1 values, but peak heights from the two amino groups in 2 are quite different due to unequal

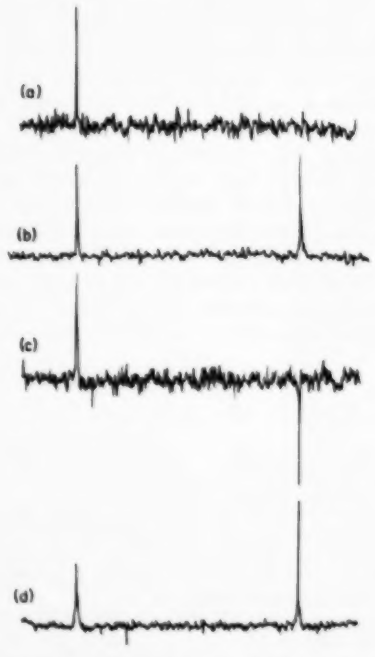

Figure 2. 2-Aminopyridine (5 M in benzene-d_6) with added paramagnetic relaxation reagents: (a) 6×10^{-2} M $Cr(acac)_3$, continuous ^{1}H decoupling; (b) same as (a) but with gated decoupling for NOE suppression; (c) 1×10^{-3} M $Gd(dpm)_3$, continuous ^{1}H decoupling; (d) same as (c) but with gated decoupling for NOE suppression. In each case 480 scans were acquired, using 90° pulses and a 5 s pulse interval.

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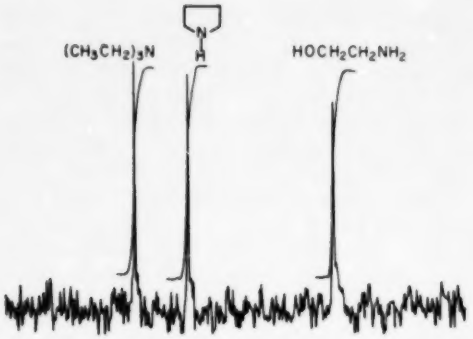

Figure 3. Quantitative ^{15}N NMR spectrum for a mixture of triethylamine, pyrrolidine, and ethanolamine (each 3 M) in benzene containing 6×10^{-2} M $Cr(acac)_3$. 800 Scans, 5 s pulse interval (45° pulses), gated decoupling.

linewidths. Only comparison of the integrals of the two lines gives correct quantitative results. In 2,4-dinitrophenol (3) both nitrogens are nonbasic, and thus paramagnetic relaxation is not a factor for these groups. Without the use of relaxation reagents, the NO_2 T_1 values are very long, lowering the efficiency of the ^{15}N experiment.

Several mixtures were prepared in order to evaluate capabilities for quantitative ^{15}N NMR analyses. An equimolar mixture of acetonitrile, dimethylformamide, and *n*-butylamine (4) was doped with 5×10^{-2} M $Cr(acac)_3$. The ^{15}N T_1 values measured in this sample were 7.2 s (CH_3CN), 5.0 s (DMF), and 4.0 s (*n*-$BuNH_2$). T_1 values were not determined in an analogous diamagnetic sample, but estimated values would be >100 s for DMF and CH_3CN, and 20 s for the amine. The T_1 values determined in the $Cr(acac)_3$-containing sample, therefore, represent effective but not quantitative replacement of diamagnetic relaxation, quenching all but minor residual Overhauser enhancements. Accumulation of signals from this mixture during an hour with NOE suppression gave satisfactory results for the content of three quite different types of nitrogen compounds. When $Gd(dpm)_3$ (1×10^{-4} M) was added to an equimolar mixture of CH_3CN, DMF and *n*-$BuNH_2$, preferential binding to *n*-butylamine was observed, as expected. In addition, both triethylamine and *n*-butylamine resonances were significantly broadened.

In another experiment a mixture of triethylamine, pyrrolidine, and ethanolamine (each 3 M) in benzene containing 6×10^{-2} M $Cr(acac)_3$ (5) was employed. T_1 values determined in this sample were 4.5 s (triethylamine), 1.6 s (pyrrolidine), and 0.6 s (ethanolamine). Figure 3 shows the result of a quantitative ^{15}N experiment for this sample employing 45° pulses and 5 s pulse intervals (total time 1.1 h).

It was hoped that a mixture containing equimolar amounts of ethanolamine, propanolamine and butanolamine (6) would have full NOEs and give good results about the composition of the mixture in a short time. Due to unequal NOEs and different linewidths, large errors were obtained in the double resonance spectrum and also from the lineheights in a gated decoupled spectrum with NOE suppression. Comparison of relaxation times from the individual compounds in the mixture shows preferential binding of paramagnetic sample impurities to ethanolamine. Selectivity in binding of paramagnetics is even observed between propanol- and butanol- amine. Treating the mixture by chelating 'Chelex' resin (Bio Rad) during 12 h with occasional shaking had only a moderate effect on freeing it from paramagnetic impurities. However, an equimolar mixture of freshly distilled ethanol- and propanol-amine (7) gave full NOEs and its composition is accurately indicated by both line intensities and by integrals. Relaxation times also show the expected pattern.

In a one-to-one mixture of *meso*- and *rac*-2,3-diaminobutanes (8) different NOEs were observed for the two isomers, demonstrating once more the very high selectivity of paramagnetic impurities on nitrogen relaxation characteristics. Only spectra taken with NOE suppression gave satisfactory quantitative results. Salts of these amines (9) had full NOEs and, in this case, quantitative data also result from the double resonance spectrum with continuous decoupling.

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